Reduced *WIF-1* Expression Stimulates Skin Hyperpigmentation in Patients with Melasma

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The expression of *Wnt inhibitory factor-1* (*WIF-1*) gene, which was detected by a microarray analysis of hyperpigmented and normally pigmented skin sets of melasma patients, was significantly reduced in the hyperpigmented skin from melasma patients, but not in healthy controls, regardless of UV irradiation. Wnt signals regulate skin pigmentation; however, *WIF-1* is expressed in cultured skin keratinocytes and fibroblasts, but not in melanocytes. Therefore, we examined whether *WIF-1* knockdown in neighboring keratinocytes and fibroblasts plays a role in melasma. Additionally, the effect of *WIF-1* overexpression on the amelioration of hyperpigmentation was examined. *WIF-1* knockdown, either in fibroblasts or in keratinocytes, significantly stimulated tyrosinase expression and melanosome transfer, whereas melanocytes with *WIF-1* overexpression significantly reduced those parameters. The *WIF-1* knockdown decreased glycogen synthase kinase-3β (GSK-3β), β-catenin, and NFATc2 (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2) phosphorylation and increased microphthalmia-associated transcription factor (MITF) expression as in melanocytes with Wnt-1 overexpression, whereas the *WIF-1* overexpression reversed the results. Expression of Wnts, both canonical and noncanonical, was increased in the hyperpigmented skin of melasma patients. Collectively, *WIF-1* downregulation, which may occur in epidermal keratinocytes and in dermal fibroblasts, is involved in melasma development because of the stimulation of melanogenesis and melanosome transfer through upregulation of the canonical and the noncanonical Wnt signaling pathway.

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INTRODUCTION

Melasma is one of the most common patterns of hyperpigmentation that affects skin on the upper lip, cheeks, forehead, and chin, particularly during the reproductive lifespan of women. In terms of pigmentation, melasma does not differ from other conditions with hyperpigmentation; however, the outcome is different from the hyperpigmentation caused by inflammation or by UV exposure. If regulatory mechanisms, which are involved in melanogenesis of these conditions, do not differ, then their outcome should not be different. Recently, we performed microarray analysis to compare hyperpigmented with normally pigmented skin specimens from patients with melasma to identify specific factors associated with the hyperpigmentation of melasma. Downregulation of H19 RNA was one of the identified factors associated with melasma (Kim *et al.*, 2010a, b). There was also a ⩾2-fold downregulation in the expression of the *Wnt inhibitory factor-1* (*WIF-1*) gene in the hyperpigmented skin of melasma patients (data not shown).

Wnts, a family of secreted glycoproteins, regulate a vast array of biological processes, including embryonic development, cell fate, cell proliferation, cell migration, stem cell maintenance, tumor suppression, and oncogenesis. In terms of pigmentation, Wnt signals have been shown to be involved in directing neural crest cells to adopt pigment cell fates (Dorsky *et al.*, 1998) and in pigment cell differentiation (Dorsky *et al.*, 2000), through either the canonical (Yamaguchi *et al.*, 2008) or the noncanonical pathways (Kim *et al.*, 2010a, b). In the canonical pathway, β-catenin plays a critical role, which is also called the Wnt/β-catenin pathway (Miller *et al.*, 1999); without the binding of canonical Wnts, such as Wnt-1, Wnt-3A, and Wnt-8, β-catenin is degraded by the destruction complex while keeping the levels of free β-catenin low. The destruction complex is composed of four proteins, and glycogen synthase kinase-3 (GSK-3) is the central player with phosphorylation of β-catenin (Yost *et al.*, 1996). With the binding to their receptor, Frizzled, these canonical Wnts prevent the degradation, which leads to a

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Abbreviations: GSK-3β, glycogen synthase kinase-3β; JNK, c-Jun N-terminal kinase; K14, cytokeratin 14; MITF, microphthalmia-associated transcription factor; NFAT, nuclear factor of activated T cells; NFATc2, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2; PKC, protein kinase C; rhWIF-1, recombinant human WIF-1; sFRP, secreted Frizzled-related protein; siRNA, small interfering RNA; TYRP-1, tyrosinase-related protein-1; WIF-1, Wnt inhibitory factor-1

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generation of a signaling pool of β-catenin, and translocate β-catenin to the nucleus. Thereby, it forms a complex with the lymphocyte enhancer-binding factor 1/T-cell factor transcription factors (Eastman and Grosschedl, 1999; Novak and Dedhar, 1999) to stimulate transcription of target genes. In addition, microphthalmia-associated transcription factor (MITF) interacts with β-catenin to activate gene expression (Schepsky *et al.*, 2006). On the other hand, noncanonical Wnt pathway is β-catenin independent and involves a diverse signal transduction, such as calcium flux, which is called the Wnt/calcium pathway, or c-Jun N-terminal kinase (JNK). In the Wnt/calcium pathway, noncanonical Wnts, such as Wnt-4, Wnt-5A, and Wnt-11, activate calcium-sensitive kinase, protein kinase C (PKC) (Sheldahl *et al.*, 1999), and calcium-responsive transcription factor, which is the nuclear factor of activated T cells (NFAT) (Murphy and Hughes, 2002; Saneyoshi *et al.*, 2002).

The extracellular antagonists of the Wnt signaling pathway can be divided into two classes (Kawano and Kypta, 2003). *WIF-1*, which is a secreted antagonist of Wnt signaling (Hsieh *et al.*, 1999), belongs to the secreted Frizzled-related protein (sFRP) class, which binds directly to Wnts in the extracellular space, along with the sFRP family. The Dickkopf class antagonists include the member of certain Dickkopf family, which inhibit Wnt signaling by binding to the low-density-lipoprotein-receptor-related protein 5 and 6 component of the Wnt receptor complex. Antagonists of the sFRP class have been considered to inhibit both canonical and noncanonical Wnt pathways, whereas those of the Dickkopf class inhibit only the canonical Wnt pathway, despite many of the unresolved issues.

Although Wnt signals regulate skin pigmentation, the expression of *WIF-1* has not been previously reported in melanocytes. On the other hand, *WIF-1* expression has been found in keratinocytes (Sasahara *et al.*, 2009; Gudjonsson *et al.*, 2010) and in fibroblasts (Cho *et al.*, 2009). Melanocyte growth and differentiation is affected by neighboring fibroblasts (Yamaguchi *et al.*, 2004) as well as by keratinocytes, and in this study we show an association between downregulation of the *WIF-1* gene in those neighboring skin cells and melasma along with its function at the molecular level. As the functional significance of *WIF-1* downregulation in cancers has been identified with *WIF-1* upregulation (Elston *et al.*, 2008; Kawakami *et al.*, 2009; Rubin *et al.*, 2010; Yee *et al.*, 2010), we used that approach to demonstrate that the upregulation of *WIF-1* using recombinant human *WIF-1* reduces pigmentation.

RESULTS

WIF-1 expression in the skin of patients with melasma

Levels of *WIF-1* mRNA expression were analyzed using real-time PCR in both hyperpigmented and normally pigmented skin specimens biopsied from 13 patients with melasma. Levels of *WIF-1* mRNA in the hyperpigmented skin were significantly ($P<0.001$) lower than in the normally pigmented skin (Figure 1a). However, the *WIF-1* mRNA levels did not reduce in the lateral cheeks or the forehead skin compared with the postauricular skin from the three normal individuals (Figure 1b). Additionally, no decrease in the *WIF-1* mRNA was observed at UV-induced hyperpigmented compared with nonexposed control skin from the abdomen of three normal individuals (Figure 1c). Double staining with antibodies to *WIF-1* and cytokeratin 14 (K14; a keratinocyte marker), which was performed in 7 of 13 patients, suggested in all cases weaker staining of *WIF-1* in the epidermal keratinocytes and in the cells that are scattered in the dermis of the hyperpigmented when compared with the normally pigmented skin specimens (Figure 1d).

WIF-1 expression in cultured skin cells

Immunohistochemistry suggested that the expression of *WIF-1* decreased in both keratinocytes and dermal cells. Melanocyte growth and differentiation is affected by neighboring keratinocytes and fibroblasts (Yamaguchi *et al.*, 2004). Therefore, expression of *WIF-1* was examined not only in melanocytes, but also in keratinocytes and in fibroblasts cultured from normal human skin. The expression of *WIF-1* mRNA and protein was detected in both keratinocytes and fibroblasts, but not in melanocytes regardless of 12-*O*-tetradecanoylphorbol-13-acetate treatment (Figure 2a and b).

Effect of *WIF-1* downregulation in keratinocytes on hyperpigmentation

After identifying that transfection of *WIF-1* small interfering RNA (siRNA) significantly ($P<0.001$) reduced the levels of both *WIF-1* mRNA and protein expression in cultured normal keratinocytes (Figure 3a), their effect was examined in keratinocyte/melanocyte co-cultures, without the purification of melanocytes. The decreased expression of *WIF-1* in the keratinocytes significantly ($P<0.005$) stimulated tyrosinase expression (Figure 3b). Concerning the signaling pathways, the *WIF-1* knockdown significantly ($P<0.05$) decreased the phosphorylation of GSK-3β and β-catenin, with increased MITF expression. The knockdown also reduced the phosphorylation of NFAT, cytoplasmic, calcineurin-dependent 2 (NFATc2) (Figure 3c). As NFATc2 and β-catenin require to translocate to the nucleus to exert their action, western blot analysis of NFATc2 and β-catenin expression performed in nuclear fraction suggested a significant ($P<0.05$) increase by the *WIF-1* knockdown (Figure 3d). The *WIF-1* knockdown also significantly ($P<0.05$) increased the proportion of tyrosinase-related protein-1 (TYRP-1) and K14 double-positive cells, which was shown in FACS analysis and confocal microscopy of co-cultured cells (Figure 3e).

Effects of *WIF-1* downregulation in fibroblasts on tyrosinase expression and melanosome transfer

As fibroblasts are components of the dermis, the effects of *WIF-1* downregulation in cultured fibroblasts were examined using a mixed-cell three-dimensional culture system, after confirming that *WIF-1* siRNA significantly ($P<0.005$) reduced the expression levels of *WIF-1* mRNA and protein in fibroblasts (Figure 4a). To keep the condition the same as the *WIF-1* downregulated keratinocytes (Figure 3b–e), the inserts containing keratinocyte/melanocyte co-cultures were used

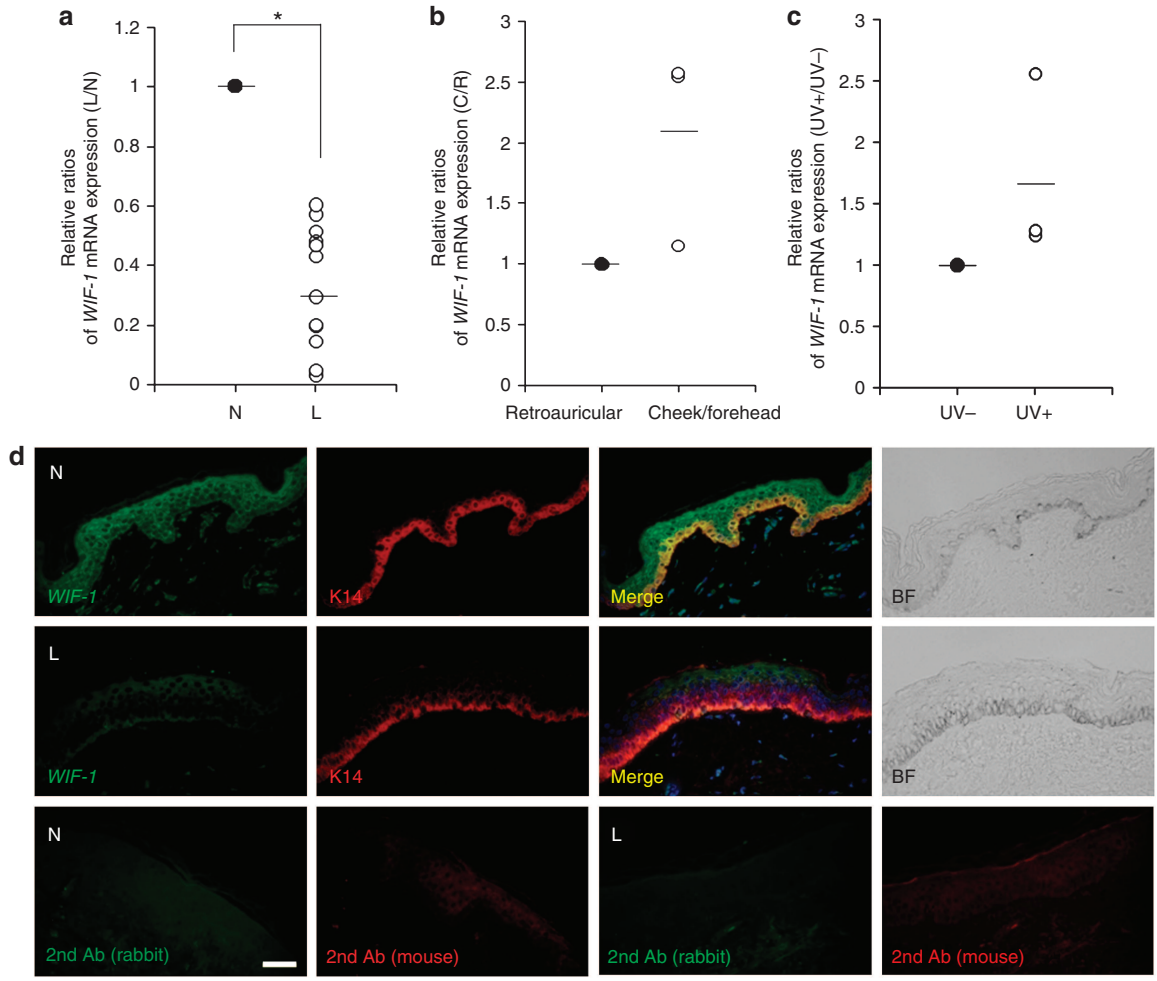

Figure 1. Expression of *Wnt inhibitory factor-1* (*WIF-1*) in the skin of patients with melasma. (**a**–**c**) Real time-PCR analysis of *WIF-1* mRNA expression in (**a**) hyperpigmented (L) and normally pigmented (N) skin specimens biopsied from 13 patients with melasma, (**b**) in corresponding paired sites, cheeks or forehead (C), and retroauricular area (R) from three healthy normal controls, and (**c**) in paired sites with (UV+) or without UV radiation (UV−) from three healthy normal controls. *$P<0.001$. (**d**) Double staining of hyperpigmented (L) and normally pigmented (N) skin of melasma patients with anti-WIF-1 and anti-K14 antibodies, which emit green and red fluorescence, respectively. Nuclei were counterstained with Hoechst 33258, which emits blue fluorescence. Staining with second antibodies only is shown for the negative control. BF, bright field; K14, cytokeratin 14. Bar = 50 μm.

for the study. Knockdown of *WIF-1* significantly ($P<0.005$) stimulated the expression levels of tyrosinase (Figure 4b). Phosphorylation of GSK-3β, β-catenin, and NFATc2 was significantly ($P<0.05$) reduced, whereas MITF expression increased (Figure 4c). FACS analysis to examine melanosome transfer to neighboring keratinocytes suggested that the percentages of TYRP-1 and K14 double-positive cells were significantly ($P<0.001$) increased following *WIF-1* knockdown (Figure 4d).

The effect of *WIF-1* upregulation on melanogenesis and melanosome transfer

The increased expression of *WIF-1* was directly induced in the monocultures of melanocytes, as well as co-cultures by the treatment with recombinant human WIF-1 (rhWIF-1; Figure 5a). Tyrosinase expression was significantly ($P<0.001$) reduced (Figure 5b) with MITF expression, whereas the phosphorylation of GSK-3β, β-catenin, and NFATc2 was increased ($P<0.05$; Figure 5c) in co-cultures. Reduced tyrosinase expression (Figure 5b) was also identified with decreased β-catenin and NFATc2 in the nuclear fraction (Figure 5d) of melanocyte monocultures. FACS analysis suggested that the proportions of TYRP-1 and K14 double-positive cells were significantly decreased ($P<0.005$; Figure 5e).

Wnts in the skin of melasma patients

WIF-1 is a secreted antagonist of Wnt signaling (Hsieh *et al.*, 1999) that inhibits both the canonical and noncanonical Wnt pathways. Our results revealed that the decreased expression of *WIF-1* in either keratinocytes or fibroblasts affected both Wnt pathways (Figures 3d and 4c), which were reversed by the *WIF-1* overexpression (Figure 5c and d). Therefore, the expression of canonical Wnts, Wnt-1 and Wnt-3A, as well as noncanonical Wnts, Wnt-5A and Wnt-11, was compared using real-time PCR in hyperpigmented and normally pigmented skin of the same 13 patients with melasma. Levels

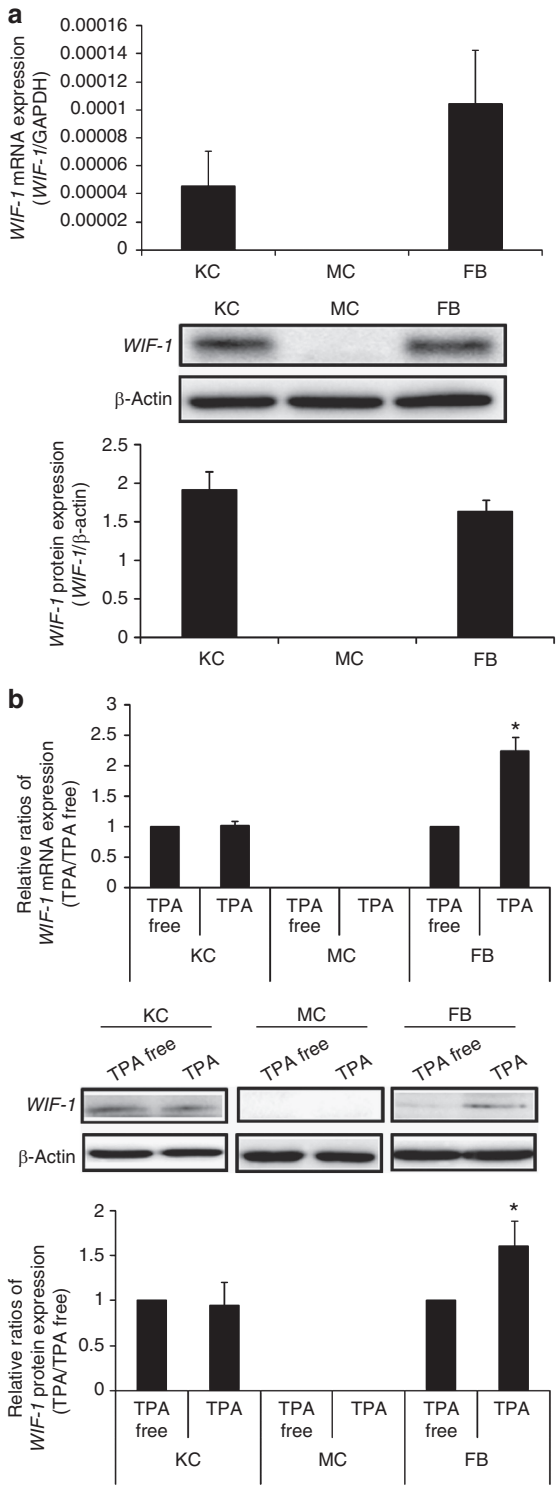

Figure 2. Expression of *Wnt inhibitory factor-1* (*WIF-1*) in cultured skin cells. Real-time PCR and western blot analysis of *WIF-1* in cultured keratinocytes (KCs), melanocytes (MCs), and fibroblasts (FBs) using medium (**a**) without or (**b**) with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Data represent means ± SD of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *$P<0.05$.

of Wnt-1 and Wnt-5A expression were significantly ($P<0.05$) higher in the hyperpigmented skin, whereas levels of other Wnts were not (Figure 6a). In fact, treatment with rhWnt-1 (Figure 6b) and rhWnt-5A (data not shown) significantly ($P<0.05$) increased the expression levels of tyrosinase with an activation of the canonical and the noncanonical pathways in cultured melanocytes.

DISCUSSION

This study reveals that decreased expression of *WIF-1* is involved in the hyperpigmented skin of patients with melasma. As *WIF-1* is an antagonist of Wnt signaling (Hsieh *et al.*, 1999), which regulates skin pigmentation, downregulation of *WIF-1* could be expected to induce hyperpigmentation of the skin. In fact, *WIF-1* mRNA levels are significantly lower in hyperpigmented skin compared with paired normally pigmented skin specimens from patients with melasma (Figure 1a), along with staining intensity of immunohistochemistry, using anti-WIF-1 antibody (Figure 1d). Although the biopsied site of normally pigmented skin is not the forehead or the cheek, but the retroauricular area, the effect of sunlight or site difference on the *WIF-1* downregulation could be excluded, because the *WIF-1* mRNA levels did not reduce in paired skin specimens of the corresponding sites and of the absence or presence of UV irradiation from healthy control individuals (Figure 1b and c). In addition, UV irradiation on cultured keratinocytes, fibroblasts, and melanocytes did not induce any change in *WIF-1* expression levels (data not shown).

Expression of *WIF-1* mRNA and protein, which was examined in cellular components of the epidermis and the dermis, was almost unrecognizable in melanocytes, whereas they were easily detected in keratinocytes and fibroblasts (Figure 2a and b), as previously reported (Cho *et al.*, 2009; Sasahara *et al.*, 2009; Gudjonsson *et al.*, 2010). The 12-*O*-tetradecanoylphorbol-13-acetate contained in the melanocyte culture medium, which was also added into the media for keratinocyte/melanocyte co-cultures, could affect the Wnt signaling pathway through the PKC activation (Niedel *et al.*, 1983). However, the *WIF-1* expression, with or without 12-*O*-tetradecanoylphorbol-13-acetate, was not changed in melanocytes and keratinocytes, although it was increased in the fibroblasts (Figure 2a and b). Therefore, we hypothesized that the downregulation of *WIF-1* function in keratinocytes and/or fibroblasts, instead of melanocytes, may have an impact on the skin hyperpigmentation. In fact, double staining, which uses antibodies to *WIF-1* and K14, suggests a weaker staining of *WIF-1* in the epidermal keratinocytes of the hyperpigmented compared with that of the normally pigmented skin specimens of melasma patients (Figure 1d). Purification of melanocytes from keratinocyte/melanocyte co-cultures required the treatment of enzymes, although that from co-cultured fibroblasts did not. Therefore, keratinocyte/melanocyte co-cultures were used to examine the effect of *WIF-1* downregulated keratinocytes or fibroblasts on normal melanocytes. As established in the cell-to-cell interactions between keratinocytes and melanocytes, the decreased expression of *WIF-1* in keratinocytes stimulates tyrosinase

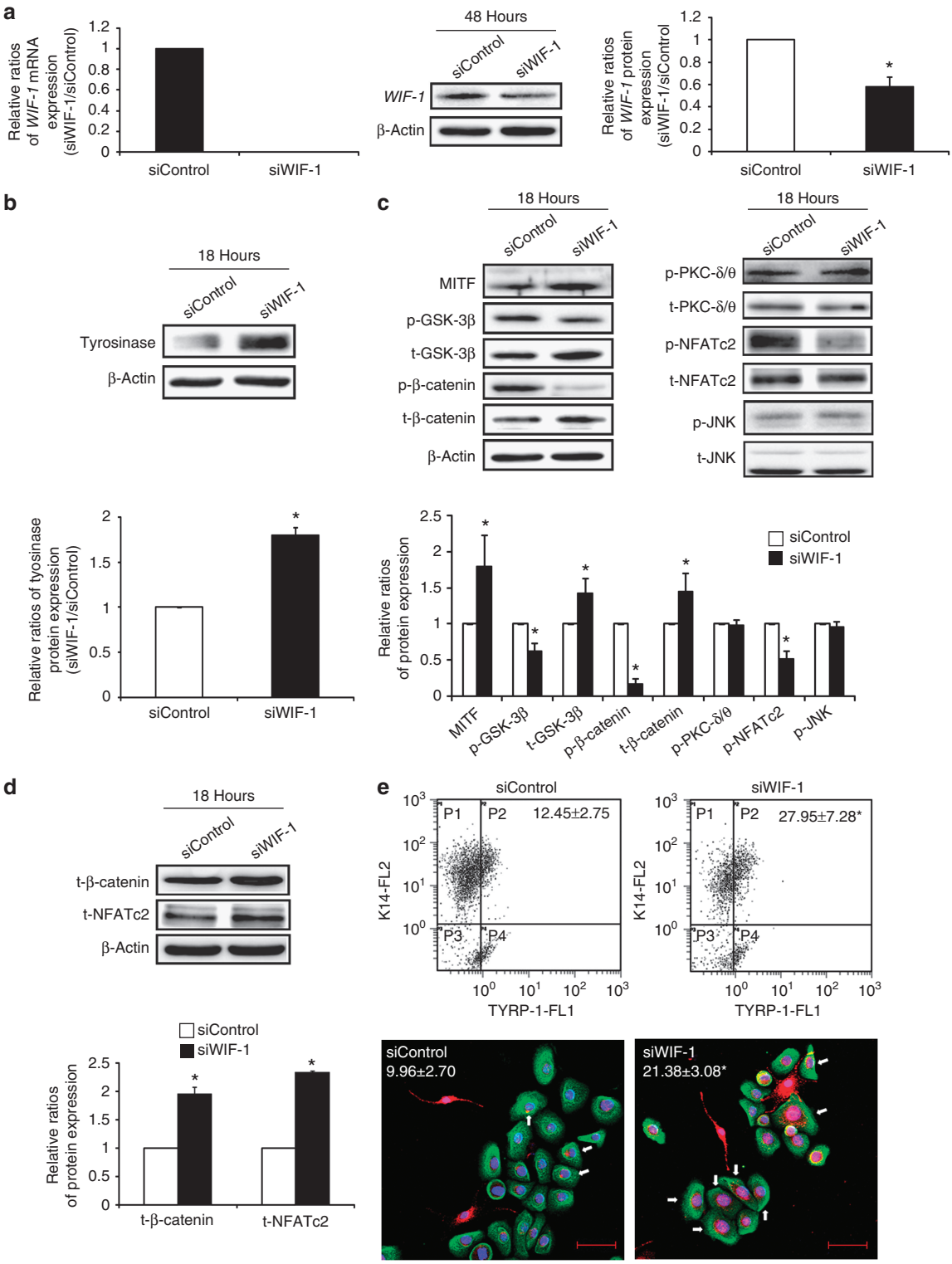

Figure 3. The effects of *Wnt inhibitory factor-1* (*WIF-1*) knockdown in keratinocytes on hyperpigmentation. (**a**) Real-time PCR and western blot analysis for the transfection of *WIF-1* siRNA (siWIF-1) and control siRNA (siControl) in keratinocytes. Data represent means ± SD of three and five independent experiments, respectively. **P*<0.001 (**b**–**d**) Western blot analysis of tyrosinase (**b**) and Wnt signaling factors in total cell lysates (**c**) and in nuclear fractions (**d**) from melanocyte/keratinocyte co-cultures in which the keratinocytes had been transfected with siWIF-1 or siControl. Data represent means ± SD of five independent experiments. **P*<0.005 and *P*<0.05 for tyrosinase and Wnt signaling factors, respectively. (**e**) FACS analysis and immunofluorescence (X–Y two-dimensional (2D) image) study using anti-TYRP-1 (red) and anti-K14 antibodies (green) with or without WIF-1 knockdown. The cells (white arrow), which contained various amount of TYRP-1-positive particles around/over the nuclei (blue), indicate positive cells. Data represent means ± SD of three independent experiments. **P*<0.05. FL (binding ability to fluorochromes) 1: FITC; FL2: phycoerythrin. GSK-3β, glycogen synthase kinase-3β; JNK, c-Jun N-terminal kinase; K14, cytokeratin 14; MITF, microphthalmia-associated transcription factor; NFATc2, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2; p, phospho; PKC, protein kinase C; siRNA, small interfering RNA; t, total; TYRP-1, tyrosinase-related protein-1. Bar = 50 μm.

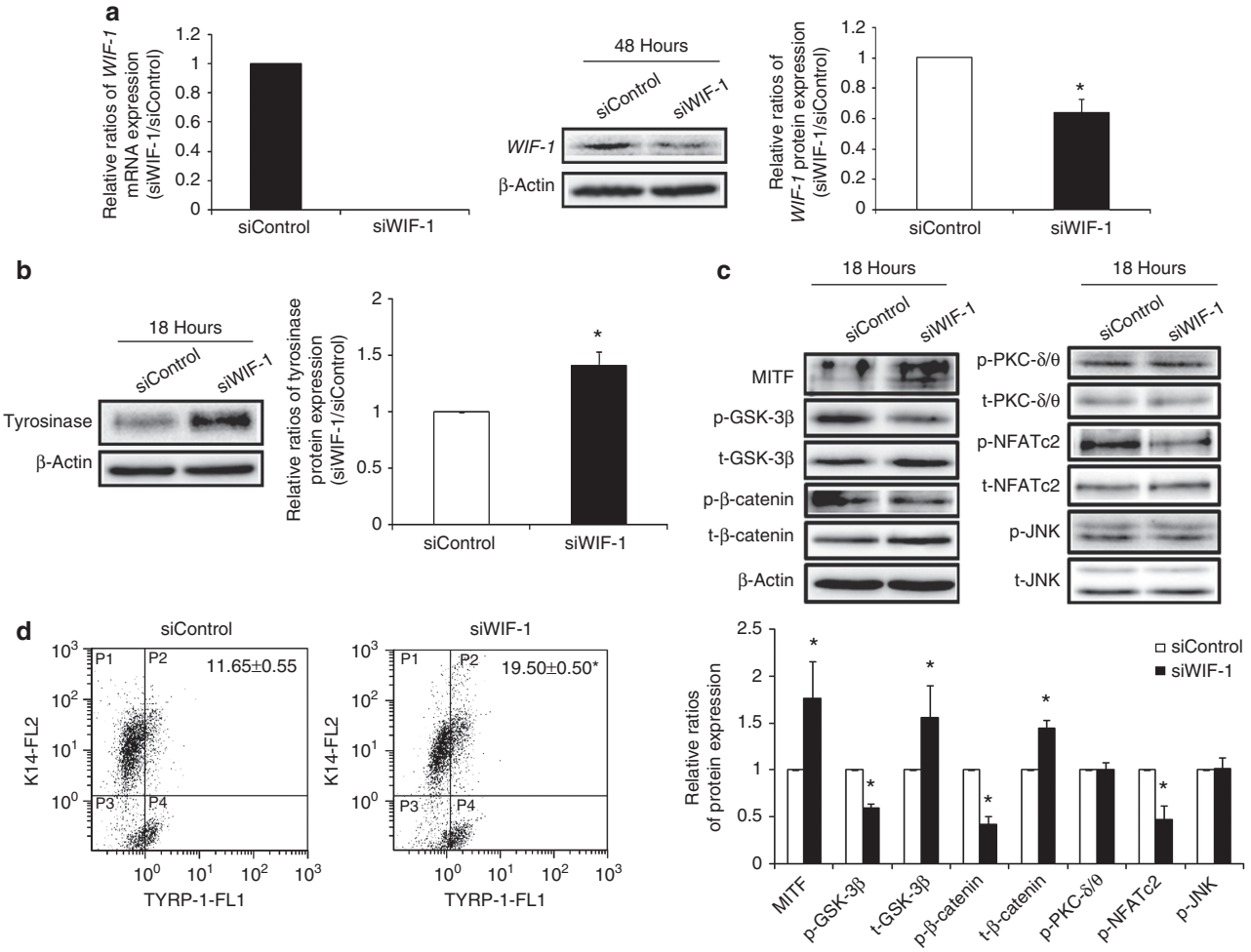

Figure 4. Effects of *Wnt inhibitory factor-1* (*WIF-1*) knockdown in fibroblasts on tyrosinase expression and melanosome transfer. (**a**) Real-time PCR and western blot analysis for the transfection of *WIF-1* siRNA (siWIF-1) and control siRNA (siControl) in fibroblasts. Data represent means ± SD of three and five independent experiments, respectively. **P*<0.005. (**b**, **c**) Western blot analysis of (**b**) tyrosinase and (**c**) Wnt signaling factors in keratinocyte/melanocyte co-cultures that were cultured three-dimensionally with fibroblasts transfected with or without *WIF-1* knockdown. Data represent means ± SD of five independent experiments.(**P*<0.005 and *P*<0.05 for tyrosinase and Wnt signaling factors, respectively. (**d**) FACS analysis using anti-TYRP-1 and anti-K14 antibodies with or without *WIF-1* knockdown. Data represent means ± SD of three independent experiments. **P*<0.001. FL (binding ability to fluorochromes) 1: FITC; FL2: phycoerythrin. GSK-3β, glycogen synthase kinase-3β; JNK, c-Jun N-terminal kinase; K14, cytokeratin 14; MITF, microphthalmia-associated transcription factor; NFATc2, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2; p, phospho; PKC, protein kinase C; siRNA, small interfering RNA; t, total; TYRP-1, tyrosinase-related protein-1.

expression (Figure 3b) and melanosome transfer from co-cultured normal melanocytes (Figure 3e). Regulation of melanogenesis could also result from complex interactions between melanogenic regulators in melanocytes and factors derived from fibroblasts. Extracellular matrix proteins derived from human dermal fibroblasts increase tyrosinase activity in normal melanocytes (Hedley *et al.*, 1996). The expression of high levels of dickkopf 1 (DKK1), an inhibitor of the canonical Wnt signaling pathway as a member of the Dickkopf class of Wnt antagonists, has also been reported in palmoplantar fibroblasts (Yamaguchi *et al.*, 2004). In our study, the effect of *WIF-1* knockdown in fibroblasts was examined using a three-dimensional collagen gel model (Yamaguchi *et al.*, 1999) to simulate real skin conditions. *WIF-1* knockdown in fibroblasts resulted in increases of both tyrosinase expression (Figure 4b) and melanosome transfer to keratinocytes (Figure 4d). Moreover, the result from the *WIF-1* knockdown in neighboring keratinocytes or fibroblasts is reversed by treatment with rhWIF-1 in melanocyte mono-cultures, as well as in keratinocyte/melanocyte co-cultures (Figure 5b and e), suggesting the significance of down-regulating *WIF-1* function on hyperpigmentation.

WIF-1 belongs to the sFRP class of Wnt antagonists, which can inhibit both the canonical and the noncanonical Wnt pathways. In this study, the decreased expression of *WIF-1*, either in keratinocytes (Figure 3c) or fibroblasts (Figure 4c), reduces phosphorylation of GSK-3β and β-catenin, which results in translocation of β-catenin to nucleus (Figure 3d). These results indicate an involvement of the canonical Wnt/β-catenin pathway with stimulating transcription of target genes. Increase in MITF expression also supports the involvement of the canonical pathway (Schepsky *et al.*,

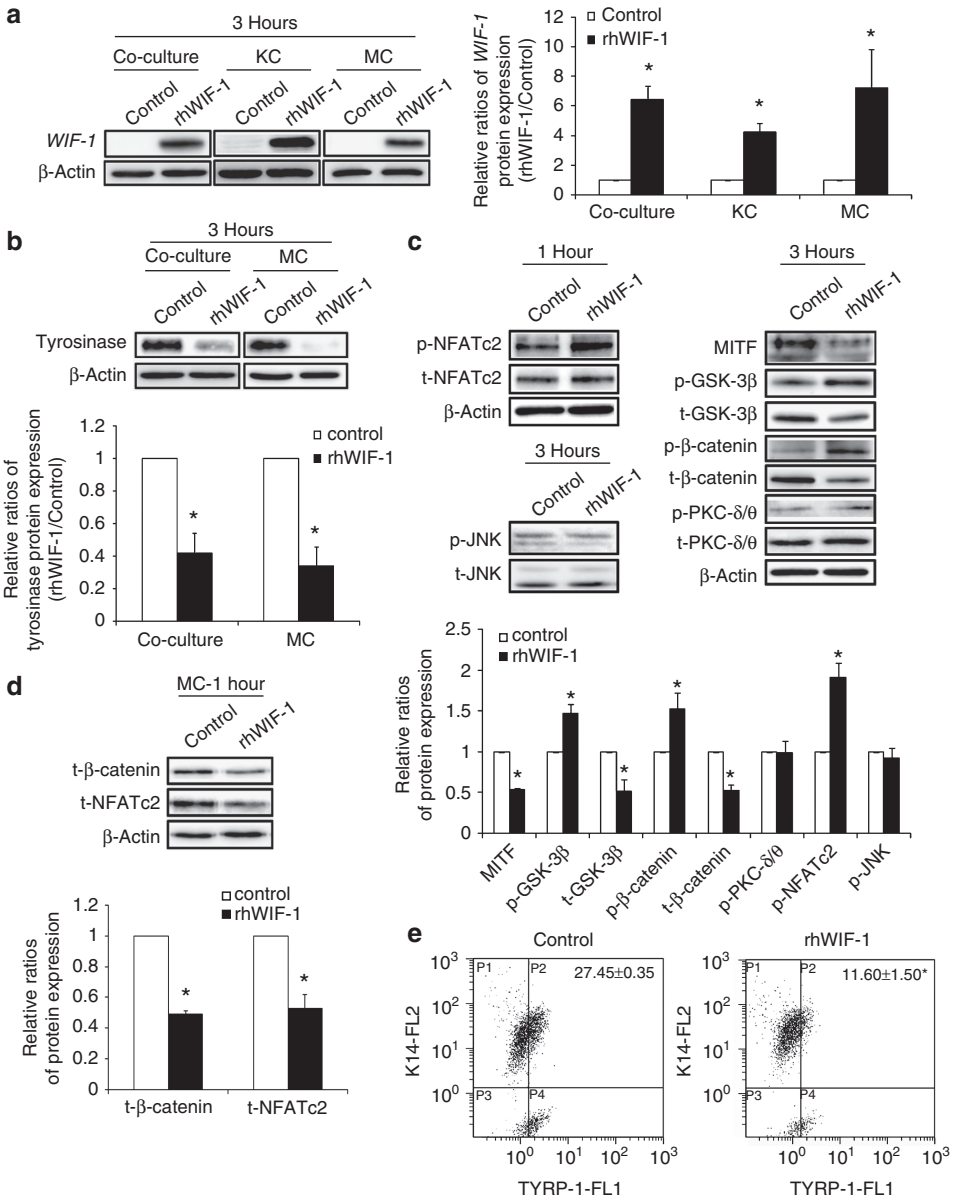

Figure 5. The effect of *Wnt inhibitory factor-1* (*WIF-1*) overexpression on melanogenesis and melanosome transfer. (**a**) Western blot analysis of *WIF-1* expression following treatment of keratinocytes (KCs), melanocytes (MCs), or keratinocyte/melanocyte co-cultures (Co-culture) with recombinant human WIF-1 (rhWIF-1). Data represent means ± SD of five independent experiments. *$P<0.05$. (**b**, **d**) Western blot analysis of (**b**) tyrosinase and (**c**) Wnt signaling factors in total cell lysates from co-cultures and/or in (**d**) nuclear fractions from monocultures of melanocytes simultaneously treated with or without rhWIF-1. Data represent means ± SD of three independent experiments. *$P<0.001$ and $P<0.05$ for tyrosinase and Wnt signaling factors, respectively. (**e**) FACS analysis using anti-TYRP-1 and anti-K14 antibodies in keratinocyte/melanocyte co-cultures treated with or without rhWIF-1. Data represent means ± SD of three independent experiments. *$P<0.005$. FL (binding ability to fluorochromes) 1: FITC; FL2: phycoerythrin. GSK-3β, glycogen synthase kinase-3β; JNK, c-Jun N-terminal kinase; K14, cytokeratin 14; MITF, microphthalmia-associated transcription factor; NFATc2, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2; p, phospho; PKC, protein kinase C; siRNA, small interfering RNA; t, total; TYRP-1, tyrosinase-related protein-1.

2006). The *WIF-1* knockdown in either keratinocytes or fibroblasts also increases the dephosphorylation of NFATc2, which results in the translocation of NFATc2 to nucleus (Figure 3d) that indicates the involvement of the noncanonical pathway with stimulating transcription of target genes. Although PKC activation responds to calcium signals (Sheldahl *et al.*, 1999) as does NFATc2 (Murphy and Hughes, 2002; Saneyoshi *et al.*, 2002), and PKC-ζ (San-Antonio *et al.*, 2002) and PKC-θ (Granja *et al.*, 2008) have interacted with NFATc2, the phosphorylation of PKC-δ/θ is not significant with the *WIF-1* knockdown (Figures 3c and 4c). The result that overexpression of *WIF-1* by rhWIF-1 treatment reverses the expression of both canonical and noncanonical signaling molecules (Figure 5c) supports the notion that *WIF-1* exerts its action through both the canonical and the noncanonical Wnt pathways. *WIF-1* overexpression decreased β-catenin and

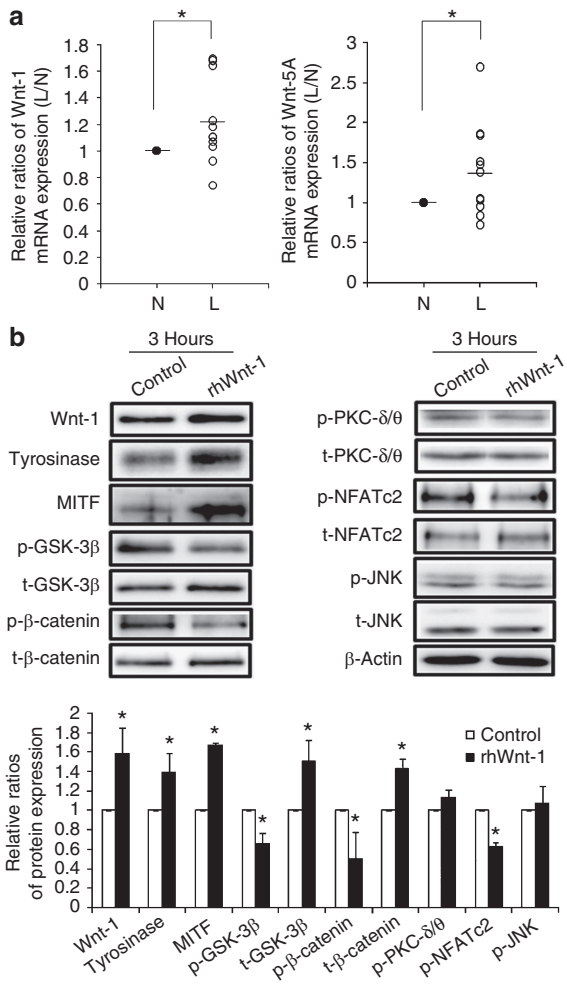

Figure 6. Expression of Wnts in the skin of melasma patients. (**a**) Real-time PCR analysis of Wnt-1 and Wnt-5A mRNA expression in hyperpigmented (L) and in normally pigmented (N) skin specimens from 13 patients with melasma. *$P<0.05$. (**b**) Western blot analysis of tyrosinase and Wnt signaling factors in cultured melanocytes treated with recombinant human Wnt-1 (rhWnt-1). Data represent means ± SD of three independent experiments. *$P<0.05$. GSK-3β, glycogen synthase kinase-3β; JNK, c-Jun N-terminal kinase; MITF, microphthalmia-associated transcription factor; NFATc2, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2; p, phospho; PKC, protein kinase C; t, total.

NFATc2 in nuclear fraction of melanocyte monocultures, as well as in total cell lysates of keratinocyte/melanocyte co-cultures (Figure 5c and d), suggesting the involvement of same pathways in melanocytes.

WIF-1 is supposed to inhibit the Wnt action by direct binding to Wnts. The increased Wnt-1 and Wnt-5A mRNA levels in patients with melasma, who had reduced *WIF-1* mRNA expression levels (Figure 6a), have supported the interaction between *WIF-1* and Wnts. Although Wnt expression in melanocytes by *WIF-1* knockdown, either in keratinocytes or in fibroblasts, was not examined, the increased melanogenesis and the involved signaling pathways by the knockdown were the same as a result of the treatment of cultured melanocytes with rhWnt-1 (Figure 6b) and rhWnt-5A. Although it is uncertain how the reduced WIF-1 expression in the neighboring keratinocytes or fibroblasts could affect the melanogenesis in melanocytes, paracrine effect could be expected, due to an unrecognizable *WIF-1* expression levels in melanocytes. Decreased *WIF-1* expression in the neighboring keratinocytes or fibroblasts could reduce *WIF-1* binding to Wnts in melanocytes, resulting in an increased Wnt expression and exerting the action through the canonical and the noncanonical Wnt pathways.

In summary, decreased expression of *WIF-1*, which may occur in epidermal keratinocytes and dermal fibroblasts, is implicated in melasma development via the stimulation of melanogenesis and melanosome transfer through the upregulation of Wnt, both canonical and noncanonical, signaling pathway.

MATERIALS AND METHODS

Patients and healthy controls

A total of 13 female patients diagnosed with melasma between 35 and 58 years of age (mean 44 years) were included in the study. The Institutional Review Board of Dongguk University Ilsan Hospital approved this study, and the study was conducted according to the Declaration of Helsinki Principles. After obtaining informed written consent from participants, skin specimens were obtained by biopsy. Pairs of normally pigmented and hyperpigmented samples were taken from each participants for direct comparisons. As the location of the hyperpigmented lesions was on the lateral side of the forehead or the upper cheek and control skin was on the retroauricular area, two corresponding sites were compared in the three age-matched healthy females. Hyperpigmented skin, which was induced by topical psoralen application, followed by a UVA irradiation twice a week for 2 weeks in abdominal skin of another group of three healthy males, was also compared.

Normal human keratinocyte, melanocyte, and fibroblast cultures

Adult skin specimens obtained from Caesarean sections and circumcisions were used to establish cells in culture.

For keratinocyte culture, individual epidermal cells were suspended in EpiLife Medium (catalog number M-EPI-500-CA; Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract, bovine insulin, hydrocortisone, human EGF, and bovine transferrin (catalog number S-001-5; Invitrogen). Keratinocytes from passages 3 or 4 were used in these experiments. For melanocytes, individual epidermal cells were suspended in Medium 254 (Invitrogen) supplemented with bovine pituitary extract, fetal bovine serum, bovine insulin, hydrocortisone, basic fibroblast growth factor, bovine transferrin, heparin, and phorbol 12-myristate 13-acetate (Invitrogen). Melanocytes at passages between 7 and 15 were used.

For fibroblast culture, individual dermal cells were suspended in DMEM (Gibco/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco/BRL), 100 U ml^{-1} penicillin (Gibco/BRL), and 0.1 mg ml^{-1} streptomycin (Gibco/BRL). Fibroblasts at passages between 5 and 10 were used.

WIF-1 knockdown

For *WIF-1* downregulation, keratinocytes or fibroblasts were cultured in six-well plates (NUNC, Roskilde, Denmark) or 100 mm

culture dishes (NUNC) and were transfected with 100 nM or 500 nM siRNA for human *WIF-1* or a negative control (ON-TARGETplus SMARTpool or Non-targeting siRNA; Dharmacon Research, Lafayette, CO) using the *Trans*IT-siQUEST transfection reagent (Mirus, PanVera, Madison, WI) according to the manufacturer's protocol.

At 24 hours after the incubation, the transfected keratinocytes were co-cultured with normal melanocytes for another 18 hours to examine the effect of keratinocytes with *WIF-1* knockdown on melanogenesis and melanosome transfer.

For the effect of fibroblast with *WIF-1* knockdown, three-dimensional cultures were performed using the collagen gel model (Yamaguchi *et al.*, 1999): the transfected fibroblasts were suspended in 1 ml of collagen matrix (Invitrogen) and cultured in six-well culture dishes (Corning, Corning, NY) for 1 hour. Then, normal keratinocytes were cultured onto BioCoat cell culture inserts (Corning) for 24 hours, before adding normal melanocytes onto the same inserts.

Treatment with rhWIF-1 or rhWnt-1

For the overexpression of *WIF-1* or Wnt-1, melanocytes and/or keratinocytes were simultaneously treated with or without 50 ng ml^{-1} rhWIF-1 (R&D Systems, Minneapolis, MN) or Wnt-1 (BioVision, Milpitas, CA) for 1 or 3 hours.

Nuclear protein extraction

Nuclear protein fraction was purified from melanocyte monocultures or keratinocyte/melanocyte co-cultures, using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo, Rockford, IL). The protein concentration of the lysate was measured using bicinchoninic acid protein assay kit (Thermo).

Real-time PCR

Levels of *WIF-1*, Wnt-1, and Wnt-5A mRNAs were quantitated by real-time PCR using the Light Cycler real-time PCR (Roche, Mannheim, Germany). Their relative amounts were calculated using the ratio of each mRNA relative to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used and probes were as follows: WIF-1 (No. HS00183662-m1; designed using Applied Biosystems software, Foster City, CA); Wnt-1 5′-CTCAT GAACCTTCACAACAACGA-3′ (forward) and 5′-ATCCCGTGGCAC TTGCA-3′ (reverse); Wnt-5A 5′-GACCACATGCAGTACATCGGA GAAG-3′ (forward) and 5′-TCCACCTTCGATGTCGGAATTG-3′ (reverse); GAPDH 5′-TCCACTGGCGTCTTCACC-3′ (forward) and 5′-GGCAGAGATGATGACCCTTT-3′ (reverse).

Western blot analysis

Equal amounts of extracted proteins (20 μg) were resolved and transferred to nitrocellulose membranes. The membranes were incubated with antibodies to β-catenin, phospho-β-catenin, GSK-3β, phospho-GSK3β, JNK, phospho-JNK, PKC-δ/θ, phospho-PKC-δ/θ, WIF-1, and NFATc2 (rabbit polyclonal; Cell Signaling Technology, Beverly, MA), phospho-NFATc2, MITF (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), tyrosinase (goat polyclonal; Santa Cruz Biotechnology), and Wnt-1 (rabbit polyclonal; Epitomics, Burlingame, CA). After incubating with appropriate anti-mouse or anti-rabbit horseradish peroxidase–conjugated antibodies (Thermo) or with anti-goat horseradish peroxidase–conjugated antibody (Santa Cruz Biotechnology) and an enhanced chemiluminescence solution (Thermo), the signals were captured on an Image Reader (LAS-3000; Fuji Photo Film, Tokyo, Japan). To monitor the amount of protein loaded in each lane, the membranes were reprobed with a mouse monoclonal anti-β-actin antibody (Sigma, St Louis, MO) and were processed as described above. The protein bands were then analyzed by densitometry.

Immunohistochemistry

Epidermal specimens were fixed in 4% paraformaldehyde, and were then dehydrated and embedded in paraffin. After deparaffinization, the sections were pretreated with a citric acid solution (100 mM citrate, pH 6.0) and 0.5% Triton X-100.

After blocking with 3% BSA, epidermal sections were incubated with anti-WIF-1 (1:100 dilution; rabbit polyclonal; Santa Cruz Biotechnology) and K14 (1:100 dilution; mouse monoclonal; Santa Cruz Biotechnology) antibodies for primary antibodies. After staining with Alexa Fluor–labeled goat anti-rabbit IgG (1:200 dilution; 488; Molecular Probes, Eugene, OR) and Alexa Fluor–labeled goat anti-mouse IgG (1:200 dilution; 594; Molecular Probes), the stained cells were observed, using a fluorescence microscope (Dp Manager 2.1; Olympus Optical, Tokyo, Japan).

Melanosome transfer

Flow cytometry analysis was performed as described previously (Lin *et al.*, 2008). Briefly, the cells were fixed with 2% paraformaldehyde, and incubated with both FITC-conjugated goat anti-TYRP-1 (1 μg per 1×10^6 cells; Santa Cruz Biotechnology) and phycoerythrin-conjugated mouse anti-K14 (1:10; BD Biosciences, San Jose, CA). Labeled cells were analyzed with a Cytomics FC500 flow cytometer (Beckman Coulter, Hialeah, FL) using CXP software (Beckman Coulter). Melanosome transfer efficacy was determined as the number of K14 and TYRP-1 double-positive cells divided by the total number of K14-positive cells.

For immunofluorescence study, the cultured cells were fixed with a 4% paraformaldehyde solution and were pretreated with 0.2% of Triton X-100. The cells were incubated with anti-TYRP-1 antibody (1:200 dilution; goat polyclonal; Santa Cruz Biotechnology) and K14 (1:200 dilution; Santa Cruz Biotechnology), and then with Alexa Fluor–labeled donkey anti-goat IgG (1:200 dilution; Molecular Probes) as well as Alexa Fluor–labeled goat anti-mouse IgG (1:200 dilution; Molecular Probes). Stained cells were observed using a Zeiss LSM 510 confocal fluorescence microscope (Oberkochen, Germany). For quantitative analysis of K14 and TYRP-1 double-positive cells, 10 random fields were photographed.

Statistical analysis

Statistical significance was assessed using analysis of variance tests. A P-value of <0.05 is considered statistically significant. All results are presented as means ± SD of the combined data from three or five independent experiments.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Cho SW, Yang JY, Sun HJ *et al.* (2009) Wnt inhibitory factor (WIF)-1 inhibits osteoblastic differentiation in mouse embryonic mesenchymal cells. *Bone* 44:1069–77

Dorsky RI, Moon RT, Raible DW (1998) Control of neural crest cell fate by the Wnt signalling pathway. *Nature* 396:370–3

Dorsky RI, Raible DW, Moon RT (2000) Direct regulation of nacre, a zebrafish MITF homolog required for pigment cell formation, by the Wnt pathway. *Genes Dev* 14:158–62

Eastman Q, Grosschedl R (1999) Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Curr Opin Cell Biol* 11:233–40

Elston MS, Gill AJ, Conaglen JV *et al.* (2008) Wnt pathway inhibitors are strongly down-regulated in pituitary tumors. *Endocrinology* 149: 1235–42

Granja AG, Perkins ND, Revilla Y (2008) A238L inhibits NF-ATc2, NF-kappa B, and c-Jun activation through a novel mechanism involving protein kinase C-theta-mediated up-regulation of the amino-terminal transactivation domain of p300. *J Immunol* 180:2429–42

Gudjonsson JE, Johnston A, Stoll SW *et al.* (2010) Evidence for altered Wnt signaling in psoriatic skin. *J Invest Dermatol* 130:1849–59

Hedley S, Gawkrodger DJ, Weetman AP *et al.* (1996) Investigation of the influence of extracellular matrix proteins on normal human melanocyte morphology and melanogenic activity. *Br J Dermatol* 135:888–97

Hsieh JC, Kodjabachian L, Rebbert ML *et al.* (1999) A new secreted protein that binds to Wnt proteins and inhibits their activities. *Nature* 398:431–6

Kawakami K, Hirata H, Yamamura S *et al.* (2009) Functional significance of Wnt inhibitory factor-1 gene in kidney cancer. *Cancer Res* 69:8603–10

Kawano Y, Kypta R (2003) Secreted antagonists of the Wnt signalling pathway. *J Cell Sci* 116:2627–34

Kim JH, Sohn KC, Choi TY *et al.* (2010a) Beta-catenin regulates melanocyte dendricity through the modulation of PKCzeta and PKCdelta. *Pigment Cell Melanoma Res* 23:385–93

Kim KH, Lee CH, Lee AY (2010b) H19 RNA downregulation stimulated melanogenesis in melasma. *Pigment Cell Melanoma Res* 23:84–92

Lin HC, Shieh BH, Lu MH *et al.* (2008) A method for quantifying melanosome transfer efficacy from melanocytes to keratinocytes *in vitro*. *Pigment Cell Melanoma Res* 21:559–64

Miller JR, Hocking AM, Brown JD *et al.* (1999) Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca2+ pathways. *Oncogene* 18:7860–72

Murphy LL, Hughes CC (2002) Endothelial cells stimulate T cell NFAT nuclear translocation in the presence of cyclosporin A: involvement of the wnt/glycogen synthase kinase-3 beta pathway. *J Immunol* 169: 3717–25

Niedel JE, Kuhn LJ, Vandenbark GR (1983) Phorbol diester receptor copurifies with protein kinase C. *Proc Natl Acad Sci USA* 80:36–40

Novak A, Dedhar S (1999) Signaling through beta-catenin and Lef/Tcf. *Cell Mol Life Sci* 56:523–37

Rubin EM, Guo Y, Tu K *et al.* (2010) Wnt inhibitory factor 1 decreases tumorigenesis and metastasis in osteosarcoma. *Mol Cancer Ther* 9:731–41

San-Antonio B, Iñiguez MA, Fresno M (2002) Protein kinase Czeta phosphorylates nuclear factor of activated T cells and regulates its transactivating activity. *J Biol Chem* 26:27073–80

Saneyoshi T, Kume S, Amasaki Y *et al.* (2002) The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos. *Nature* 417:295–9

Sasahara Y, Yoshikawa Y, Morinaga T *et al.* (2009) Human keratinocytes derived from the bulge region of hair follicles are refractory to differentiation. *Int J Oncol* 34:1191–9

Schepsky A, Bruser K, Gunnarsson GJ *et al.* (2006) The microphthalmia-associated transcription factor Mitf interacts with beta-catenin to determine target gene expression. *Mol Cell Biol* 26:8914–27

Sheldahl LC, Park M, Malbon CC *et al.* (1999) Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. *Curr Biol* 9:695–8

Yamaguchi Y, Itami S, Tarutani M *et al.* (1999) Regulation of keratin 9 in nonpalmoplantar keratinocytes by palmoplantar fibroblasts through epithelial-mesenchymal interactions. *J Invest Dermatol* 112:483–8

Yamaguchi Y, Itami S, Watabe H *et al.* (2004) Mesenchymal-epithelial interactions in the skin: increased expression of dickkopf1 by palmoplantar fibroblasts inhibits melanocyte growth and differentiation. *J Cell Biol* 165:275–85

Yamaguchi Y, Passeron T, Hoashi T *et al.* (2008) Dickkopf 1 (DKK1) regulates skin pigmentation and thickness by affecting Wnt/beta-catenin signaling in keratinocytes. *FASEB J* 22:1009–20

Yee DS, Tang Y, Li X *et al.* (2010) The Wnt inhibitory factor 1 restoration in prostate cancer cells was associated with reduced tumor growth, decreased capacity of cell migration and invasion and a reversal of epithelial to mesenchymal transition. *Mol Cancer* 9:162–75

Yost C, Torres M, Miller JR *et al.* (1996) The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes Dev* 10:1443–54